Isolated and purified human soluble guanylyl cyclase α1/β1 (hsGC α1/β1)

The technical field of the invention

The present invention relates to the expression of the cDNA clones for the subunits α1 (hsGCα1) and β1 (hsGCβ1) of human soluble guanylyl cyclase and the subsequent purification of the active enzyme and the use thereof, the medical application of the expression of these clones by gene transfer, as well as antibodies to peptides derived from the sequence and the use thereof.

State of the art

The endogenous NO/cGMP signaling system mediates important functions such as vasodilation, inhibition of platelet aggregation, neurotransmission, and the immune response. In addition, it is involved in the development of various disease states such as ischemia-reperfusion and inflammatory injuries (Schmidt and Walter, 1994). Therefore, the NO/cGMP system has long been an important starting point for the development of novel drugs for the therapy of coronary heart disease, susceptibility to thrombosis, cardiac insufficiency, angina pectoris, cardiac-dependent pulmonary oedema, hypertensive crises, inflammatory states and cardiac infarction. Until now, such therapies have employed various so-called NO donors, e.g. nitroglycerin, that release NO, thereby replacing endogenous NO and activating soluble guanylyl cyclase (sGC) (Fig. 1). sGC forms cGMP, which mediates the effects of the NO/cGMP pathway via various intracellular receptor enzymes. The application of NO donors has two limitations: I) The repeated application of NO donors results in tolerance in the patient, i.e. loss of activity, when reapplied; II) NO reacts with O_2^- to form peroxynitrite, which is cytotoxic and less effective in activating sGC. Thus, direct activation of sGC by novel, non-NO-containing pharmaceuticals for therapy or gene transfer of sGC would be desirable strategies. Non-NO-releasing activators of sGC are potentially lacking tolerance and NO toxitication. Recently, "YC-1" (3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazol) has been described as the first non-NO-releasing activator of sGC in platelets (Ko et al., 1994; Wu et al., 1995). YC-1 also

activates purified sGC from bovine lung and potentiates the activation of NO (Friebe et al., 1996). The effect on human sGC has not been investigated. The human isoform of sGC is not yet available for pharmacological screening.

The NO receptor sGC consists of two subunits, α and β, which together form an enzymatically active heterodimer. Three different isoforms of the α and three of the β subunits have been described in the literature, although from different species. The best investigated isoforms, the bovine α1/β1 and the rat α1/β1 isoforms, have particular significance for cardiovascular research. Until recently, a human homologue of the bovine and rat sGC (e.g. sGCα1/sGCβ1 heterodimers) was unknown. cDNA sequences have been published that reportedly correspond to a human sGCα3 and β3 isoform (Giuili et al., 1992). While sGCβ3 showed a high homology to sGCβ1 (bovine/rat), the sGCα3 sequence contained two restricted regions without homology to sGCα1 (bovine/rat), here designated S1 and S2 (Fig. 2). In addition, it was unknown whether sGCα3 and sGCβ3 can form a functional sGC heterodimer and which role the S1 and S2 regions play. Furthermore, no other human sGC subunit has yet been expressed as a protein. Recently, a sequence designated hsGCα1 was published in the GeneBank (Accession No. U58855) that lacks the sequence differences to sGCα1 from bovine and rat tissues in the regions S1 and S2. In addition, an alternatively spliced product of hsGCβ3 was recently published in the GeneBank (Accession No. AF020340) that was designated by the authors as an alternatively spliced form of hsGCβ1. The physiological significance of this splice variant of hsGCβ1/3 is unclear. Thus, the question arises as to which of these isoforms is responsible for which physiological function in which cell types.

Furthermore, no antibodies to the human sGCα1/β1 are currently available that are monospecific, directed against the human sequences, or that have been shown to be suitable for immunoblots with human tissues. Peptide antibodies reported thus far only partially show these features: Harteneck et al. and Guthmann et al. used a peptide sequence (VYKVETVGDKYMTVSGLP) that is highly conserved in guanylyl cyclases. Therefore, cross-reaction with particulate guanylyl cyclases (e.g. GC-C) can be expected. Guthmann et al. used a peptide sequence (YGPEVWEDIKKEA) identical to hsGCβ1 and a peptide sequence (KKDVEEANANFLGKASGID) identical

to hsGCα1 except for two amino acid exchanges. However, the function of these antibodies in immunoblots was only shown for *enriched* hsGC from human platelets. In addition, the antisera to hsGCα1 detect a second, unspecific product. Humbert et al. and Koesling et al. used a peptide sequence (SRKNTGTEETEQDEN) of bovine sGCβ1 that partially (amino acids 1-10) overlaps with the peptide used here for the human sGCβ1 (amino acids 13-22) and is identical in this region. The C-terminus of this bovine peptide (amino acids 11-15), however, is clearly different from the human sequence. On the other hand, the antiserum to this peptide was not tested with a human protein but rather only used for immunoprecipitation of the bovine sGC.

Thus, for the human isoform α1/β1 which is important for cardiovascular research, neither *native* protein nor *recombinant* protein, nor a one-step purification protocol, nor specific antibodies have been available. An approach for gene therapy (e.g. with adenoviruses or the like) has also not been described yet.

The technical problem of the invention

The NO-independent activation of the human sGCα1/β1 is a promising approach to finding novel drugs or gene transfer techniques for cardiovascular therapy that neither result in tolerance in the patient nor form cytotoxic peroxynitrite. To find such pharmaceuticals, a mass screening for suitable active substances is necessary. Such a pharmacological screening for specific activators or inhibitors via animal testing is too expensive and is not reasonable due to species differences, possible side effects, and effects on other isoforms. Cell culture systems have the disadvantage that only with substantial additional effort can it be determined at which points of the signaling cascade substances are effective. In addition, cell culture is expensive and touchy. The purification of a protein from animal tissues is labor-intensive and results in a low yield. More importantly, because of species differences the results of such a screening are not generally transferable. Here, in particular, the question arises of the significance of the isoforms hsGCα1 and hsGCα3 and whether hsGCβ3 in fact corresponds to a human hsGCβ1. This is an important factor to consider in selecting the appropriate target protein for pharmacological screening or gene therapy. For optimal screening, the human homologue of the protein sGCα1/β1 would have to be

purified in large amounts and to be available at low cost. A purification of large amounts of human native sGCα1/β1 protein from human tissues is not possible. Thus, other methods are needed for the drug screening protocol. In addition, no purified antibodies are available for the detection of human sGCα1/β1 that have been shown to be suitable for diagnostic uses, e.g. in normal immunoblotting, ELISA, RIA, or EIA, among other techniques. An approach for the artificial expression of hsGC by gene transfer in humans that could be used therapeutically is also not available.

Therefore, the present invention is based on the technical problem of providing isolated and purified human sGCα1/β1 as well as a process for its production and purification. In addition, a further technical prblem of the present invention is to provide antibodies directed against human sGCα1/β1. A further technical problem is the provision of expression vectors containing the cDNA of human sGCα1/β1 based on adenoviruses. Finally, the technical problem of the present invention is the provision of human sGCα1/β1 in purified form and in manageable amounts for drug screening assays aimed at detecting modulators, inhibitors, and activators of human sGCα1/β1.

The solution of the technical problem

The solution to the above technical problem is provided by the subject-matter of the claims and the following description of the invention.

An object of the invention is a human soluble guanylyl cyclase α1/β1 (hsGCα1/β1) in an isolated form purified to apparent homogeneity.

Another object of the invention is a process for the production of subunit α1 and/or β1 of human soluble guanylyl cyclase comprising the expression of expression vectors containing the DNA sequence for hsGCα1 and/or hsGCβ1 in prokaryotic and eukaryotic host cells, and obtaining of the subunit or subunits.

In a preferred embodiment of the process of the present invention for the production of subunit α1 and/or β1 of human soluble guanylyl cyclase, the step of obtaining the

subunit or subunits comprises the lysis of cells, affinity chromatography of the cell lysate, and finally, the elution of the subunit or subunits.

In a further preferred embodiment of the process of the present invention for the production of subunit α1 and/or β1 of human soluble guanylyl cyclase, the expression vector contains additionally at least one DNA sequence of a domain for specific affinity chromatography (affinity tag) with attached protease cleavage site.

Another object of the present invention is a process for the production of human soluble guanylyl cyclase α1/β1 (hsGCα1/β1) comprising the separate expression of an expression vector containing the DNA sequence for hsGCα1 or hsGCβ1 in prokaryotic or eukaryotic host cells, obtaining the subunits, and combining the subunits hsGCα1 and hsGCβ1 into the dimeric guanylyl cyclase α1/β1 (hsGCα1/β1).

A further preferred embodiment of the process of the present invention for the production of human soluble guanylyl cyclase α1/β1 (hsGCα1/β1) also comprises the coexpression of the DNA sequences for hsGCα1 and hsGCβ1 in prokaryotic or eukaryotic host cells, the lysis of the cells containing hsGCα1 and hsGCβ1, affinity chromatography, and the subsequent elution of hsGCα1/β1.

Another object of the present invention is the use of a nucleic acid sequence that codes for subunits hsGCα1 and/or hsGCβ1 of human soluble guanylyl cyclase α1/β1 for somatic gene therapy, particularly for the prevention and therapy of atherosclerosis and its complications, restenosis, ischemia (infarction), peripheral occlusive diseases, and arterial hypertension, as well as, in patients with risk factors, for the prevention of atherosclerosis, transient ischemic attacks, cerebral ischemia, stroke, coronary heart disease, status post coronary bypass grafting, carotid stenosis, heart insufficiency and liver dysfunction, and as a supplement to therapy with sGC activators, sGC-sensitizing substances, NO donors, or phosphodiesterase inhibitors.

In a particularly preferred embodiment, adenoviral vectors containing hsGCα1 or hsGCβ1 cDNA are used in somatic gene therapy. Other vector systems, however, can also be applied for gene therapeutic, medicinal use.

In a preferred embodiment, adenoviral Ad5 and other suitable vectors that contain the nucleic acid sequence of human soluble guanylyl cyclase α1 (hsGCα1) and/or human soluble guanylyl cyclase β1 (hsGCβ1) are used for prevention and therapy of the diseases mentioned above. Likewise, a mixture of two vectors in which one vector contains the nucleic acid sequence of human soluble guanylyl cyclase α1 (hsGCα1) and the second that of human soluble guanylyl cyclase β1 (hsGCβ1) can be used for somatic gene transfer. Especially preferred is somatic gene transfer into endothelial cells, vascular smooth muscle cells, neointimal cells, fibroblasts, and other vascular cells as well as into blood particles (platelets, leukocytes, and others) and the liver.

The methods of gene transfer described in the present invention can also be used for gene transfer of human soluble guanylyl cyclase α2 (GeneBank: x63282) and of the human homologue of soluble guanylyl cyclase β2 (from rat; GeneBank: m57507) as well as for other human soluble guanylyl cyclases.

Another object of the present invention relates to antibodies to human soluble guanylyl cyclase α1/β1 (hsGCα1/β1), obtainable by immunization of a mammal with hsGCα1/β1, either of subunits α1 or β1, or immunogenic peptide fragments thereof, as well as isolation of the antibodies.

Figures:

Figure 1 shows various possibilities of modulation of soluble guanylyl cyclase (CsGC-α1/sGC-β1). Normal activation is mediated by NO synthase (NOS) and NO. NO, however, reacts with oxygen radicals to form peroxynitrite ($ONOO^-$), which is cytotoxic and only poorly activates sGC. NO can also be released by NO donors such as nitroglycerin or sodium nitroprusside. sGC can be directly activated by modulators of sGC (e.g. YC-1); alternatively, the activation by NO of sGC is potentiated by these modulators. Additionally, sGC can be overexpressed by use of gene transfer (e.g. using adenoviral vectors) or a pathologically low expression level of sGC can be compensated. Adenoviral (or other) vectors with mutated sGC that

has a higher basal activity can also be used. Thus, an elevated cGMP level could be achieved permanently independent of NOS, NO, NO donors, or sGC modulators.

Figure 2 shows a schematic comparison of the bovine and rat sGCα1 subunits together with the published sequence of the human cDNA clone termed 'sGCα3' (Giuili et al., 1992). The bars represent the protein. 'N' represents the N-terminus and 'C' the C-terminus. The functional segments 'regulatory domain', 'sGC homology domain', and 'cyclase domain' of these proteins are marked with different patterns. The regions 'S1' and 'S2' for which no homologous regions are found in the bovine and rat sGCα1 proteins are marked in black.

Figure 3 shows a schematic illustration of the human sGCα3 clone with the sequence errors published (Giuili et al., 1992). The cDNA is shown above: the bar represents the coding region of the cDNA, the lines left and right of the bar represent the 5' and 3' untranslated regions, respectively. 'S1' and 'S2' represent the regions that have no homology to the bovine and rat isoforms of sGCα1 (see Fig. 2). The positions of the sequence errors are marked below: line a shows the nucleotide insertions, line b the deletion, and line c the exchanges. A base-pair (bp) scale is shown below. The sequence errors for each of the 3 lines a, b, and c are listed at the bottom: the letter specifies the type of the base concerned, and the number its position in the cDNA.

Figure 4 shows the verification of the expression of human sGCα1 (A) and sGCβ1 (B) in human tissues by means of PCR using cDNA libraries. Shown is a photo of an ethidium bromide-stained agarose gel under UV light with separated PCR products. The arrow on the left points to the specific product. The PCR primers are visible at the bottom of the photo. The tissues from which the cDNA libraries were produced are indicated at the top. No cDNA was added in the negative control, and in the positive control, plasmid containing the cDNA of hsGCα1 was added.

Figures 5 and 6 show the baculovirus transfer vectors pVL1393 and pAcG2T, respectively (both without the hsGC cDNA), which were used for the construction of recombinant baculoviruses for the expression of human sGCα1/β1 in Sf9 cells. The circular plasmid with the restriction sites (short names and position in base pairs), the gene for ampicillin resistance (Amp^R), the 'origin of replication' (ColE ori), the

polyhedrin promotor, the glutathione-S-transfease sequence (only in Fig. 6), and the multiple cloning site (MCS) are shown at the top of the figures. Figure 5 shows the multiple cloning site with its unique restriction sites at the bottom. Figure 6 shows the multiple cloning site with the unique restriction sites as well as a thrombin cleavage site normal at the bottom.

Figure 7 shows the construction of the plasmids hsGCβ1-pVL1393 (without GST-tag) with the hsGCβ1 cDNA, which was used to obtain genetically modified, hsGCβ1-expressing baculovirus by homologous recombination. The procedure for the plasmid pAcG2T-hsGCβ1 (with GST-tag = glutathione-S-transferase cDNA from *Schistosoma japonicum*) is identical. A fragment was produced that carries an additional BamHI-site at its 5' end by means of PCR with the primers A (bases 89-116 of the hsGCβ1 cDNA + BamHI site at its 5' end) and B (bases 692-711 of the hsGCβ1 cDNA [noncoding strand] with natural KpnI site). Due to the additional restriction sites, fragment 1 (PCR fragment with new BamHI site) and fragment 2 (hsGCβ1 cDNA from KpnI site to EcoRI site) could be inserted together into the BamHI and EcoRI sites of the plasmid pVL1393. Thus, the complete hsGCβ1 cDNA is under the control of the polyhedrin promotor (PHP).

Figure 8 shows the construction of the plasmid hsGCα1-pVL1393 (without GST-tag) with the hsGCα1 cDNA which was used to obtain genetically modified, hsGCα1-expressing baculovirus by homologous recombination. The procedure for the plasmid pAcG2T-hsGCα1 (with GST-tag = glutathione-S-transferase cDNA from *Schistosoma japonicum*) is identical. A fragment was produced that carries an additional BamHI site at its 5' end and a natural BsaAI site within the sequence by means of PCR with the primers C (bases 524-541 of the hsGCα1 cDNA + BamHI site at its 5' end) and D (bases 1232-1249 of the hsGCα1 cDNA [noncoding strand]). Due to the added restriction site, fragment 3 cut with BsaAI (PCR fragment with new BamHI site to the BsaAI site) and fragment 4 (hsGCα1 cDNA from BsaAI site to EcoRI site) could be inserted together into the BamHI and EcoRI sites of the plasmid pVL1393. Thus, the complete hsGCα1 cDNA is under the control of the polyhedrin promotor (PHP).

Figure 9 shows the verification of the expression of hsGCα1/β1 in Sf9 cells, which were infected with the genetically modified viruses described above (with hsGCα1 or hsGCβ1 cDNA; both without GST-tag = glutathione-S-transferase cDNA from *Schistosoma japonicum*). On the left (A) a Coomassie-stained 10% SDS-polyacrylamide gel is shown on which were loaded the cell homogenates that had been separated into pellet (P) and supernatant (S) by centrifugation (20,000 x g). 'Co' designates the control with noninfected Sf9 cells. 'α1' disignates Sf9 cells that were infected with viruses containing the hsGCα1 cDNA, and 'β1' designates Sf9 cells that were infected with viruses containing the hsGCβ1 cDNA. The position of hsGCα1 and hsGCβ1 in the gel are marked (α1 or β1). On the right an immunoblot is shown with supernatant (S) and pellet (P) of the cell homogenate from Sf9 cells that were either noninfected (Co) or coinfected with hsGCα1 and hsGCβ1 baculoviruses (α1+β1). The peptide antibodies to hsGCβ1 described above (anti-hsGCβ1) were used in immunoblotting first (Fig. 9B. lines 1-4). Afterwards, the blot was redeveloped with the peptide antibodies to hsGCα1 (anti-hsGCα1; Fig. 9B, lanes 5-8), which additionally revealed the hsGCβ1 bands.

Figure 10 shows the activity of guanylyl cyclase (formation of cGMP from GTP) in intact Sf9 cells that were coinfected with the genetically modified baculoviruses containing hsGCα1 and hsGCβ1 cDNA described here (both without GST-tag). Shown is the content of cGMP in pmol per 10^6 cells with different treatments, indicated at the bottom of the figure. Sample 1 is untreated in both panels A and B. 1 mM IBMX (3-isobutyl-1-methylxanthine) was added to each of the other samples (top line: black crossbar). In the middle line, the concentration of SNP added to the samples is indicated in µM. The bottom line shows the concentration of added YC-1 (A, left) or ODQ (B, right) in µM.

YC-1 = 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazol;

ODQ = 1H-[1,2,4]oxadiazolo[4,3,-a]chinoxalin-1-on;

SNP = sodium nitroprusside;

cGMP = cyclic 3', 5'-guanosine monophosphate

Figure 11 shows the activity of guanylyl cyclase (formation of cGMP from GTP) in homogenates of Sf9 cells that were coinfected with the genetically modified baculoviruses containing hsGCα1 and hsGCβ1 cDNA described here (both without

GST-tag). As shown at the top, (A), the soluble cell fraction (supernatant after centrifugation at 20,000 x g) was used, and at the bottom, (B), the respective pellet. In each case, the amount of cGMP formed in pmol per mg protein per minute is shown for homogenates of cells that were harvested at different time points (indicated in hours) after infection with the baculoviruses. cGMP formation was measured with (black boxes) and without (white boxes) the addition of 100 µM SNP.

Figure 12 shows the activity of guanylyl cyclase (formation of cGMP from GTP) in Sf9 cells that were coinfected with the genetically modified baculoviruses containing hsGCβ1 cDNA (without GST-tag) and hsGCα1 cDNA (with GST-tag = glutathione-S-transferase cDNA from *Schistosoma japonicum*). The formation of cGMP in pmol per mg protein per minute during the purification procedure (affinity chromatography on glutathione sepharose 4B) is shown. In each case, activity was measured in the lysate (after removing the insoluble part by centrifugation at 20,000 x g), in the supernatant after binding of hsGC to glutathione sepharose 4B (flow-through), in both supernatants of the washes of hsGC bound to glutathione sepharose 4B (1. and 2. wash), as well as in the supernatant after elution of hsGC with reduced glutathione (1. and 2. elution). The formation of cGMP was determined without (black boxes, 'basal') and with (grey boxes, '+ 100 µM SNP') addition of 100 µM SNP.

Figure 13 shows the natural, endogenous expression of hsGCα1 and hsGCβ1 in different human tissues in an immunoblot.
The peptide antibodies to hsGCα1 described above (anti-hsGCα1) were used on the left (A), and the peptide antibodies to hsGCβ1 (anti-hsGCβ1) were used on the right (B). On the right, the peptide to which the antibodies were raised was added as negative control (peptide: +), while no peptide was added on the left (peptide: -). The SDS extracts of rhsGCα1- (in panel A of the figure) or rhsGCβ1-overexpressing Sf9 cells (in panel B of the figure) (Sf9), of human cerebral cortex (cortex), of human cerebellum (cerebellum), and of human lung (lung) were loaded on an 8% polyacrylamide gel. The specific bands of hsGCα1 (α1) and hsGcβ1 (β1) are indicated by an arrow.

Figure 14 shows in immunoblots the purification of hsGCα1 (as a hsGCα1/hsGCβ1 dimer) from Sf9 cells that were coinfected with the genetically modified baculoviruses

containing hsGCα1 cDNA with GST-tag (= glutathione-S-transferase cDNA from *Schistosoma japonicum*) and hsGCβ1 cDNA (without GST-tag). Cell lysate was incubated with glutathione sepharose 4B, and after binding, the supernatant was loaded (supernatant after binding). The sepharose was washed twice and the respective supernatants of this wash were loaded (1. and 2. wash). Subsequently, elution was performed by cleavage of the hsGCα1 protein from the GST-tag with thrombin and an aliquot was loaded ('elution with thrombin').Then, SDS stop buffer was added to the glutathione sepharose 4B and an aliquot was loaded (GSH sepharose after elution). In addition, glutathione sepharose 4B with bound hsGCα1 without prior thrombin elution to which SDS buffer had been added was loaded (GSH sepharose before elution). The immunoblot was developed with the affinity-purified peptide antibodies to the C-terminus of hsGCα1 described here. The arrows on the right point to the specific bands of hsGCα1 with the GST-tag (GST-hsGCα1) and hsGCα1 without the GST-tag (hsGCα1).

Figure 15 shows the purification of hsGCα1/β1 from Sf9 cells that were coinfected with the genetically modified baculoviruses containing hsGCα1 cDNA with GST-tag (= glutathione-S-transferase cDNA from *Schistosoma japonicum*) and hsGCβ1 cDNA without GST-tag in a Coomassie Brilliant Blue R250-stained SDS-polyacrylamide gel. Cell lysate of these infected Sf9 cells (lysate) was incubated with glutathione sepharose 4B, and the supernatant loaded after the binding (supernatant after binding). The glutathione sepharose 4B was washed twice and the respective supernatants of this wash buffer were loaded (1. and 2. wash). In one sample, the bound GST-hsGCα1/β1 was eluted by incubation with reduced glutathione and loaded (elution with glutathione). In the other samples, the glutathione sepharose was washed with the buffer for the thrombin cleavage—without thrombin—and the supernatant of this buffer was loaded (3. wash). Then, the hsGCα1/β1 dimer was eluted by incubation with different amounts of thrombin and the eluates were loaded (elution with 0.25-1 U/ml thrombin) [U = unit]. The same relative amount of each of the samples was used. The bands visible with the different elution methods are indicated on the right: GST-hsGCα1 = hsGCα1 with GST-tag; hsGCα1 = hsGCα1 without GST-tag; hsGCβ1 = hsGCβ1 without GST-tag. On the left are the molecular

weight standards loaded on the gel, the size of which (in kDa) is indicated on the far left side.

Figure 16 shows the construction of the recombinant adenoviral hsGC vectors. The cDNAs of hsGCα1 and hsGCβ1 (grey bars) were inserted in the adenoviral transfer plasmid pZS2, which has a deletion in the adenovirus E1 region (ΔE1) and a unique XbaI site in this plasmid. This resulted in the plasmids hsGCα1-pZS2 and hsGCβ1-pZS2, respectively. hsGCα1-pZS2 and hsGCβ1-pZS2 cut with the restriction enzyme XbaI (middle bar, indicated as 'sGCpZS2') were ligated into the XbaI site of the long arm (upper bar, 'RR5') of Ad5. This resulted in the adenovectors Ad5CMVhsGCα1 and Ad5CMVhsGCβ1, respectively (lower bar, 'Ad 5 CMV sGC') in which the sGC cDNAs lie under the control of the CMV promotor and CMV enhancer (CMV = cytomegalovirus).

Figure 17 shows the stimulation of sGC activity by 100 µM SNP (= sodium nitroprusside) in EA.hy926 cells that were coinfected with both hsGC adenoviruses Ad5CMVhsGCα1 and Ad5CMVhsGCβ1 (samples A-C) and in noninfected EA.hy926 cells (sample D). The amount of pmol cGMP formed per mg protein per minute is plotted on the Y axis. The dark bars represent basal cGMP formation without SNP stimulation, and the light bars represent cGMP formation with SNP stimulation.

Figure 18 shows the DNA sequence of human soluble guanylyl cyclase α1 (hsGCα1); SEQ ID NO: 1.

Figure 19 shows the amino acid sequence of human soluble guanylyl cyclase α1 (hsGCα1); SEQ ID NO: 2.

Figure 20 shows the DNA sequence of human soluble guanylyl cyclase β1 (hsGCβ1); SEQ ID NO: 3.

Figure 21 shows the amino acid sequence of human soluble guanylyl cyclase β1 (hsGCβ1); SEQ ID NO: 4.

Figure 22 shows the amino acid sequence of the peptide that was used for the production of antibodies to human soluble guanylyl cyclase α1 (hsGCα1) (corresponds to amino acids 634-647 of hsGCα1); SEQ ID NO: 5.

Figure 23 shows the amino acid sequence of the peptide that was used for the production of antibodies to human soluble guanylyl cyclase β1 (hsGCβ1) (corresponds to amino acids 593-614 of hsGCβ1); SEQ ID NO: 6.

Figure 24 shows the DNA sequence of the PCR primer pair for human soluble guanylyl cyclase α1 (hsGCα1). Upper primer (corresponds to nucleotides 524-541 of the hsGCα1 cDNA sequence with added BamHI restriction site); SEQ ID NO: 7. Lower primer (corresponds to nucleotides 1249-1232 of the hsGCα1 cDNA sequence [noncoding strand]); SEQ ID NO: 8.

Figure 25 shows the DNA sequence of the PCR primer pair for human soluble guanylyl cyclase β1 (hsGCβ1). Upper primer (corresponds to nucleotides 89-106 of the hsGCβ1 cDNA sequence with added BamHI restriction site); SEQ ID NO: 9. Lower primer (corresponds to nucleotides 692-711 of the hsGCβ1 cDNA sequence [noncoding strand]); SEQ ID NO: 10.

Figure 26 shows the stimulation of sGC activity by 100 μM SNP (sodium nitroprusside) in ECV 304 cells that had been coinfected with different amounts of both hsGC adenoviurses Ad5CMVhsGCα1 and Ad5CMVhsGCβ1 (samples 1-6) and in noninfected ECV 304 cells (sample 7). The amount of pmol cGMP formed per mg protein per minute is plotted on the Y axis. The dark bars represent basal cGMP formation without SNP stimulation, and the light bars represent cGMP formation with SNP stimulation.

Figure 27 shows the stimulation of sGC activity by 100 μM SNP (sodium nitroprusside) in A10 cells that had been coinfected with different amounts of both hsGC adenoviurses Ad5CMVhsGCα1 and Ad5CMVhsGCβ1 (samples 1-6) and in noninfected A10 cells (sample 7). The amount of pmol cGMP formed per mg protein per minute is plotted on the Y axis. The dark bars represent basal cGMP formation

without SNP stimulation, and the light bars represent cGMP formation with SNP stimulation.

Figure 28 shows the stimulation of sGC activity by 100 µM SNP (sodium nitroprusside) in ECV 304 cells that had been coinfected with 5×10^{10} of each of the hsGC adenoviruses Ad5CMVhsGCα1 and Ad5CMVhsGCβ1 and then have been harvested at different time points as well as the activity in noninfected ECV 304 cells. The time point of the harvest, in days after infection (0, no infection), is plotted on the X axis. The amount of pmol cGMP formed per mg protein per minute is plotted on the Y axis. The dark bars represent basal cGMP formation without SNP stimulation, and the light bars represent cGMP formation with SNP stimulation.

Figure 29 shows the stimulation of sGC activity by 100 µM SNP (sodium nitroprusside) in A10 cells that had been coinfected with 5×10^{10} of each of the hsGC adenoviruses Ad5CMVhsGCα1 and Ad5CMVhsGCβ1 and then have been harvested at different time points as well as the activity in noninfected A10 cells. The time point of the harvest, in days after infection (0, no infection), is plotted on the X axis. The amount of pmol cGMP formed per mg protein per minute is plotted on the Y axis. The dark bars show basal cGMP formation without SNP stimulation, and the light bars represent cGMP formation with SNP stimulation.

Figure 30 shows the detection of sGC subunits in E304 cells and A10 cells, respectively, that had been coinfected with 5×10^{10} of each of the hsGC adenoviruses Ad5CMVhsGCα1 and Ad5CMVhsGCβ1 for two days in a protein immunoblot with the peptide antibodies to hsGCα1 and hsGCβ1 described above. Lane 1, purified hsGC (according to Figure 12); lane 2, ECV 304 cells with hsGC gene transfer; lane 3, A10 cells with hsGC gene transfer; lane 4, purified hsGC (according to Figure 12).

Advantages of the present invention and solution of the above-mentioned technical problems according to the present invention

1.) The cDNA clones described in the literature as sGCα3 and sGCβ3 were identified as human homologues of bovine and rat sGCα1 and sGCβ1, and in the following are termed human sGCα1 (hsGCα1) and human sGCβ1 (hsGCβ1). According to present knowledge, this sGCα1/β1 isoform is pharmacologically more important due to its function in the cardiovascular system. Because the original clone of hsGCα3 was examined, it could be shown that hsGCα1 and hsGCα3 do not exist in parallel but rather only the form hsGCα1. Thus, an obvious target protein for pharmacological mass screening and gene therapy has been identified.

2.) With the methods described by the present invention, a functional, active expression of human sGC has been obtained for the first time. Thus, the respective protein can be produced by genetic methods for the first time.

3.) By use of the peptide antibodies to sGC of the present invention, it is possible to determine the expression of sGC in human tissues as well as diagnose dysfunctional conditions (if expression of sGC is too high, too low, or absent). In addition, the present invention provides the technical prerequisites needed to further elucidate the control of transcription and translation of hsGC. The peptide antibodies of the invention have the advantage that they are monospecific, directed at the human sequence, and that their suitability for immunoblots with human tissues has been demonstrated. Other peptide antibodies exhibit these features only partially: Harteneck et al. and Guthmann et al. used a peptide sequence (VYKVETVGDKYMTVSGLP) that is relatively highly conserved in guanylyl cyclases. Thus, cross-reaction with particulate guanylyl cyclases (e.g. GC-C) would be expected. Furthermore, Guthmann et al. used a peptide sequence (YGPEVWEDIKKEA) identical to hsGCβ1 and a peptide sequence identical to hsGCα1 except for two amino acid exchanges (KKDVEEANANFLGKASGID), but the function of these antibodies in immunoblotting has only been shown for enriched hsGC from human platelets. In addition, these antibodies to hsGCα1 recognized a second, unspecific product.

Humbert et al. and Koesling et al. used a peptide sequence (SRKNTGTEETEQDEN) from bovine sGCβ1 that is in part (amino acids 1-10) identical to the peptide used here (amino acids 13-22) for hsGCβ1, although the C-terminus (amino acids 11-15) differed markedly from that of the human sequence. The antiserum to this peptide, however, has not been tested on human protein and has only been used for immunoprecipitation of bovine sGC.

In addition to the peptides shown in Figures 22 and 23 and their immunogenic fragments for the production of the antibodies to hsGCα1 or hsGCβ1 in rabbits of the present invention, the production of monoclonal or polyclonal antibodies to the whole hsGCα1/β1 protein or its cleavage products is possible. Various animal species (preferably mouse, rat, or rabbit) can be used for the production of these antibodies.

4.) By use of the eukaryotic baculovirus/Sf9 expression system of the present invention, human soluble guanylyl cyclase α1/β1 can be produced in high amounts. The attachment of a nucleotide sequence that codes for a polypeptide suitable for affinity chromatography (affinity tag, e.g. glutathione-S-transferase = GST-tag) with an attached protease cleavage site at the N-terminus of the cDNA of the α1 subunit allows the rapid and simple purification of the coexpressed dimeric protein by means of a single affinity chromatographic step. The attached affinity tag is subsequently removed by digestion with a protease. Thus, a protein identical in primary structure to the native protein is obtained. This revolutionary, fast, and clean purification yielding high amounts of very pure, functional human sGC results in new possibilities for a mass screening for specific activators and inhibitors as well as for the pharmacological characterization of potential drugs.

Purification of hsGCα1/β1 can also be accomplished by ion exchange chromatography, gel filtration, immunoaffinity chromatography, and other chromatographic procedures, e.g. on ATP-, GTP-. cGMP- or Blue-sepharose, and other similar chromatographic media.

5.) The process of the present invention can also be used for other isoforms of the human, rat, and bovine enzyme in an identical manner. In the process provided

in the present invention, various affinity tags (e.g. histidine oligomer) and different expression systems (e.g. *E. coli*) can be used. Other parts of the hsGCα1 and hsGCβ1 sequences can also be used for the production of antibodies to peptides or the whole protein.

6.) The availability of high amounts of an isolated human protein in high purity and in high quality is essential for modern pharmaceutical development. Until now, this requirement was not fulfilled in the search for alternatives to the classical NO donors. Mass screening for specific activators or inhibitors in animal testing is too expensive and would not make sense due to species differences, possible side effects, and effects on other isoforms. Cell culture systems have the disadvantage of requiring substantial additional effort to determine precisely at which point within the signaling cascades substances act. In addition, cell culture is expensive and touchy. Compared with the expression of a protein using recombinant DNA technology, the purification of a protein from animal tissues is more labor intensive and results in lower yields. In particular, the results of a pharmacological screening can only be generalized to a limited extent because of differences between species. In contrast, processes of the present invention provide for recombinant, inexpensive hsGCα1/β1 in high amounts, and unequivocal predictions concerning modulation, activation, or inhibition of this particular human enzyme can be made from a screening procedure. Species differences and lack of applicability to human are excluded a priori by the use of a human enzyme.

In addition, hsGC is available thereby in high amounts and adequate purity for crystallization and clarification of its structure. Thus, an important prerequisite for rational drug design by means of molecular modeling is fulfilled.

7.) In addition to the use of isolated hsGCα1/β1, intact Sf9 cells that express hsGCα1/β1 by being infected with the recombinant baculoviruses described here can be used in in vitro experiments.

8.) Transient (e.g. with adenoviral vectors) or stable overexpression can be achieved by means of gene transfer. Thus, the cGMP level can be elevated even at low

NO concentration or in the case of poor activation of sGC due to peroxynitrite formation. This approach also has conceptional advantages compared to the gene transfer of NOS (NO synthase) because the formation of cytotoxic peroxynitrite, which is less effective in activating sGC, is circumvented with the approach of the present invention. Furthermore, a permanent increase in the cGMP level could be achieved (e.g. for therapeutic purposes) independently of NOS, NO, NO donors, or sGC modulators by gene transfer of mutated sGC with elevated basal activity.

9.) In addition to the purification procedure for hsGCα1/β1 described here, purification from Sf9 cells is also possible after infection with the described baculoviruses containing hsGCα1 or hsGCβ1 cDNA if coinfection is performed with hsGCα1 baculoviruses without a GST-tag (GST-tag = appended glutathione-S-transferase sequence from *Schistosoma japonicum*) and hsGCβ1 baculoviruses having a GST-tag as well as with hsGCα1 baculoviruses having a GST-tag and hsGCβ1 baculoviruses having a GST-tag.

Furthermore, use of a column containing glutathione sepharose 4B is possible in addition to the use of glutathione sepharose 4B alone in a batch-wise step.

Purification can also be performed by digesting the dimeric fusion protein GST-hsGCα1/β1 with thrombin *after* elution of the fusion protein from the glutathione sepharose 4B with reduced glutathione. After dialysis (to remove the reduced glutathione), the GST-tag which has been cleaved from the protein can be removed from the mixture by additional affinity chromatography on glutathione sepharose 4B.

10.) The process of the present invention can also be used for human soluble guanylyl cyclase α2 (GeneBank: x63282) and any potentially existing human homologue of the soluble guanylyl cyclase β2 (from rat; GeneBank: m57507) as well as for other human soluble guanylyl cyclases (in all technical variations described in the present invention).

11.) Using the adenoviral, somatic gene transfer of hsGCα1 or hsGCβ1 by means of coinfection with Ad5CMVhsGCα1 or Ad5CMVhsGCβ1, respectively, it was demonstrated in endothelial (ECV 304) and smooth muscle (A10) cells that the intracellular sGC activity measured as cGMP content after stimulation of sGC with SNP was increased from undetectable levels ($\leq$ 100 pmol/mg/min) to 12-fold higher values. An optimal concentration, regarding the amount of adenovirus (pfu) as well as an equimolar ratio of both adenoviruses (based on pfu), corresponding to the homodimeric structure of the enzyme, was determined. This effect on intracellular sGC activity was still detectable 15 days after a single infection, a period sufficient for the intended applications.

The examples illustrate the present invention.

Example 1

The corrected sequence of hsGCα1 and hsGCβ1

The original clone of the human isoform sGCα3 and sGCβ3 (Giuili et al., 1992) was sequenced again. While the sequence of the sGCβ3 clone was confirmed (see SEQ ID NO: 3 and Figure 20), the sequencing of sGCα3 showed that the original publication (Giuili et al., 1992) contained 19 sequencing errors, which are summarized in Figure 3. The corresponding corrected α3-cDNA sequence is shown in SEQ ID NO: 1 and Figure 18. The deduced amino acid sequence is shown in SEQ ID NO: 2 and Figure 19. Furthermore, the corrected sequence (see SEQ ID NO: 1 and Figure 18) is identical with the human sGCα1 sequence published in the GeneBank (accession No. U58855), whereby the 5' untranslated region of the sequence provided here is 506 base pairs longer. Therefore, 'sGCα3' is now classified as human sGCα1 (hsGCα1). Thus, it was shown that two different hsGCα subunits α1 and α3 do not both exist in humans, which could be important for cardiovascular research but rather—in analogy to the situation in bovine and rat tissues—only hsGCα1.

Table 1 Revised terminology of soluble guanylyl cyclase cDNAs and proteins and their detection in human tissue.

	human sGC subunits	
	α	β
isoform 1	cDNA and protein detectable	cDNA and protein detectable
	active, when coexpressed	
isoform 2	cDNA detectable	
	active, when coexpressed with bovine ß1	

The expression of sGCα1 and sGCβ1 mRNA in human tissues was demonstrated by means of PCR (Figure 4). The amplification of a hsGCβ1 fragment with a PCR primer pair (5'-AAAAGGATCCATGTACGGATTTGTGAAT-3' = nucleotides 89-106 of the hsGCβ1 cDNA sequence with added restriction site; 5'-ATGCGTGATTCCTGGGTACC-3' = 692-711 of the hsGCβ1 cDNA sequence) with an annealing temperature of 54°C resulted in one specific band each in cDNA libraries from brain, heart, kidney, lung, pancreas, and skeletal muscle. The identity of the amplified fragment was confirmed by sequencing. The amplification of a hsGCα1 fragment with a PCR primer pair (5'-AAAAGGATCCATGTTCTGCACGAAGCTC-3' = nucleotide 524-541 of the hsGCα1 cDNA sequence with added restriction site; 5'-ATTATGGAAGCAGGGAGG-3' = 1249-1232 of the hsGCα1 cDNA sequence) with an annealing temperature of 54°C resulted in one specific band each in cDNA libraries from heart (Figure 4A) and lung (not shown). In each case, the sequencing of the fragments resulted in the corrected hsGCα1 sequence; and the 'hsGCα3' sequence published by Giuili et al. was not found. Thus, it was shown that in humans, only one hsGCα1/β1 exists, and that the potential hsGCα3/β3 is a result of sequencing errors. This results in a clear picture for cardiovascular research concerning which sGC isoform should be the target protein for pharmacological screening.

Example 2

Construction of recombinant baculoviruses for the expression of human sGCα and sGCβ in insect cells

In order to verify that hsGCα1 and hsGCβ1 can form a functional, heterodimeric sGC protein, both cDNAs were inserted into baculoviruses. Using these baculoviruses, the recombinant protein was expressed under the control of the strong polyhedrin promotor in insect cells (Sf9 cells). For the production of the recombinant baculoviruses, the baculovirus transfer vector pVL1393 (Pharmingen, San Diego, California, USA; Figure 5) and the baculovirus transfer vector pAcG2T (with glutathione-S-transferase sequence from *Schistsoma japonicum* and thrombin cleavage site; Pharmingen; Figure 6) were used in which the foreign genes hsGCα1 and hsGCβ1 were cloned, respectively. The cotransfection of such a recombinant pVL1393 or pAcG2T plasmid with BaculoGold baculovirus DNA (Pharmingen) allowed the direct isolation of the genetically modified baculoviruses with hsGCα1 or hsGCβ1 cDNA formed by homologous recombination from the cell culture media.

The construction of pVL1393-hsGCβ1 is schematically shown in Figure 7 (identical procedure for pAcG2T-hsGCβ1). The coding region of the hsGCβ1 cDNA with the 3' untranslated region but without the 5' untranslated region was cloned into pVL1393. For this, a BamHI site was introduced by means of PCR with primers A and B immediately upstream of the codon coding for the start methionine. The thus amplified fragment 1 was digested with BamHI/KpnI; fragment 2 was isolated from the sGCβ1 cDNA clone with KpnI/EcoRI. Fragments 1 and 2 as well as the vector opened with BamHI and EcoRI were ligated (see Figure 7).

The construction of pVL1393-hsGCα1 is shown schematically in Figure 8 (identical procedure for pAcG2T-hsGCα1). The coding region of the hsGCα1 cDNA with the 3' untranslated region but without the 5' untranslated region was cloned into pVL1393. For this, a BamHI site was introduced by means of PCR with primers C and D immediately upstream of the codon coding for the start methionine. The thus amplified fragment 3 was digested with BamHI/BsaAI; fragment 4 was isolated from

the sGCα1 cDNA clone with BsaAI/EcoRI. Fragments 3 and 4 as well as the vector opened with BamHI and EcoRI were ligated (see Figure 8).

For the production of the recombinant hsGCα1 and hsGCβ1 baculoviruses, the baculovirus transfer vectors (pVL1393-hsGCα1, pAcG2T-hsGCα1, pVL1393-hsGCβ1, pAcG2T-hsGCβ1) were each cotransfected with baculovirus DNA (Baculo-Gold; Pharmingen, San Diego, California, USA) in monolayers of Sf9 cell cultures. For this, the cells were cultivated at 27°C in IPL-41 media (Gibco) supplemented with 10% (vol/vol) fetal calf serum (Biochrom), 4% (vol/vol) tryptose-phosphate-broth (Gibco), 1% (vol/vol) Pluronic F68 (Gibco), 0,5% amphotericin B (Gibco), 80 µg/ml gentamycin sulfate (Gibco), and 0.5 mM δ-aminolevulinic acid (Merck). Recombinant hsGCα1 and hsGCβ1 baculovirus clones were obtained from the culture media by means of plaque purification. For the production of virus stock solutions with high titer, Sf9 shaking cultures (0.5 x 10^6 cells/ml) were infected with a M.O.I. (multiplicity of infection) of 0.1 pfu/cell (pfu = plaque forming units) and harvested 6 days after infection.

Example 3

Production of recombinant hsGCα1 and hsGCβ1 in Sf9 cells

Ten of each recombinant hsGCα1 and hsGCβ1 baculovirus clones were tested for expression of recombinant protein in Sf9 cells. For this, Sf9 monolayer cell cultures were infected with plaque-purified, recombinant hsGCα1 or hsGCβ1 baculoviruses, incubated at 27°C for 5 days, harvested with a cell scraper, resuspended in 0.5 ml lysis buffer (25 mM TEA, pH 7.8, 50 mM NaCl, 1 mM EDTA, 5 mM DTT, 1 µM leupeptin, 0.5 mg/l trypsin inhibitor), and homogenized by sonication ('Sonifier 250', 'standard'-tip, Branson; 15 times, 'duty cycle': 15%, intensity: 1). After centrifugation of the homogenates at 20,000 x g, the supernatant and pellet were analyzed by SDS-PAGE. Three of the hsGCβ1 baculovirus clones and two of the hsGCα1 baculovirus clones yielded recombinant protein in amounts that were visible in the insoluble fraction by staining with Coomassie Brilliant Blue R250. Recombinant human sGCα and sGCβ (rhsGCα and rhsGCβ) migrated with an apparent molecular weight of 79.5 (hsGCα1) and 68.5 kDa (hsGCβ1), which is very close to the predicted molecular

weights deduced from the amino acid sequence (77.5 and 70.5 kDa, respectively) (representative clones in Figure 9). The baculovirus clones that showed the highest expression of recombinant proteins in the immunoblot were used for the expression of functional heterodimeric hsGC [see examples 4-7].

Example 4

Recombinant human sGC in intact insect cells is active and stimulated by NO

For the production of functional heterodimeric human sGC, rhsGCα1 and rhsGCβ1 were coexpressed in Sf9 cells with recombinant baculoviruses. For this, Sf9 monolayer cultures [2.5 x 10^6 cells/dish, Ø 90 mm; supplements see example 2] were coinfected with a M.O.I. (multiplicity of infection) of 2 pfu/cell of each recombinant baculovirus (hsGCα1 and hsGCβ1; both without GST-tag) and cultivated for 48 hours at 27°C. The basal as well as the NO-stimulated activity of the sGC in the cells was determined by measurement of the cGMP content in the cells in the presence of the phosphodiesterase inhibitor IBMX (3-isobutyl-1-methylxanthine).

For the determination of the cGMP content, the culture media was replaced by Krebs-Ringer-buffer (KRB; 119 mM NaCl; 4.74 mM KCl; 2.54 mM $CaCl_2$; 1.19 mM $MgSO_4$; 1.19 mM KH_2PO_4; 25 mM $NaHCO_3$; 10 mM HEPES; pH 7.4; 0.1% BSA) which, in addition, contained 1 mM IBMX. The cells were incubated for one hour at 27°C. Then the cells were washed with ice-cold KRP and harvested in 1 ml ice-cold ethanol (80 %) with a cell scraper. The cells were homogenized by sonication [see Example 3] and centrifuged at 20,000 x g for 20 minutes. The supernatant was dried in a speed-vac and the residue resuspended in 25 mM TEA, pH 7.8. The cGMP content was determined by means of RIA (Biotrend).

The coexpression of rhsGCα1 and rhsGCβ1 resulted in the formation of functional sGC with basal activity in Sf9 cells (Figure 10): While noninfected Sf9 cells contained approximately 0.1 pmol cGMP/10^6 cells (not shown), approximately 20 pmol cGMP/10^6 cells was found in rhsGC-expressing cells (Figure 10). This basal activity of recombinant hsGC was increased by an NO donor, SNP (sodium nitroprusside). When the cells were incubated with 10, 100, or 1000 µM SNP for 2 minutes prior to

the harvest, the cGMP content increased in a concentration-dependent manner by up to 50 fold (Figure 10).

Example 5

Recombinant human sGC in insect cell extracts is active and stimulated by NO

The activity of the recombinant hsGC (after expression with the recombinant baculoviruses described above) was not only determined in intact Sf9 cells but also in Sf9 cell extracts. For the production of such extracts, Sf9 shaking cultures [2 x 10^6 cells/ml; supplements see Example 2] were coinfected with a M.O.I. (multiplicity of infection) of 1 pfu/cell of each virus (hsGCα1 and hsGCβ1; both without GST-tag) and incubated at 27°C. Samples were taken (4 ml) at 0, 24, 48, 72, 96, and 118 hours after infection, the cells sedimented, resuspended in 1 ml lysis buffer and homogenized by sonication [see Example 3]. The homogenates were centrifuged at 20,000 x g for 15 minutes and the insoluble pellet was resuspended again in lysis buffer. The samples were adjusted to 50% glycerin (vol/vol) and stored at –20°C. The protein concentration was determined spectrophotometrically with the standard method of Bradford (Bradford, 1976). The sGC activity was determined by the formation of [^{32}P]cGMP from [α^{32}P]GTP (Schultz and Böhme, 1984). The reactions contained 50 mM TEA (pH 7.4), 3 mM $MgCl_2$, 3 mM DTT, 1 mM IBMX, 1 mM cGMP, 5 mM creatine phosphate, 0.25 mg/ml creatine kinase, and 500 µM GTP in a total volume of 100 µl. After incubation at 37°C for 10 minutes, the reaction was started by simultaneous addition of cell extract and the sGC activators SNP, CO, or YC-1. The [^{32}P]cGMP formed was measured as described (Schultz and Böhme, 1984).

Basal rhsGC activity (i.e. cGMP formation by rshGC *without* activation of the enzyme by addition of NO or other activators), mainly found in the soluble Sf9 cell fraction (Figure 11A), reached is maximum 72 hours after infection of the cells and was increased up to 5 fold with 100 µM SNP (Figure 11A). The pellet fraction did not contain measurable basal sGC activity at any time point; although in the presence of SNP a low degree of sGC activity was found (Figure 11B).

Example 6

Influence of YC-1 and ODQ on recombinant human sGC

YC-1 (3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazol) and ODQ (1H-[1,2,4]oxadiazol[4,3,-a]chinoxalin-1-on) are substances that were described to specifically influence the activity of sGC. Thus, it was investigated if this also holds true for rhsGC.

After expression with the recombinant baculoviruses (without GST-tag) described above, rhsGC was activated in intact Sf9 cells by YC-1. The NO-potentiating effect was also found: the cGMP content of cells expressing rhsGC was increased 3.4 fold by incubation with 10 µM YC-1 for 2 minutes (Figure 10A). 100 µM YC-1 had the same effect (Figure 10A). When the cells were simultaneously treated with YC-1 and 100 µM SNP, the cGMP levels doubled compared to the cGMP levels after stimulation with SNP alone (Figure 10A). Similar results were obtained with rhsGC in cell extracts.

ODQ is described as a selective inhibitor of NO-stimulated sGC that, however, does not inhibit the basal activity (Garthwaite et al., 1995). In Sf9 cells expressing rhsGC (after expression with the recombinant baculoviruses described above, without GST-tag), ODQ did not have an influence on the basal cGMP levels; the stimulation of rhsGC in intact cells with SNP, however, was inhibited by simultaneous incubation with ODQ (Figure 10B).

Example 7

Extraction of the purified human soluble guanylyl cyclase α1/β1 (hsGCα1/β1)

For the purification of recombinant human hsGCα1/β1 from Sf9 cells, a recombinant baculovirus [see Example 2] was used in which a fusion protein composed of hsGCα1 and attached GST [so-called GST-tag, GST = glutathione-S-transferase from *Schistosoma japonicum*; see Example 2] is formed. Using this GST-tag, which binds to glutathione with high affinity, specific affinity chromatography on glutathione sepharose 4B (Pharmacia, Freiburg, Germany) can be performed. The Sf9 cells coinfected with hsGCα1 baculoviruses (with GST-tag) and hsGCβ1 baculoviruses (without GST-tag) were lysed in 25 mM triethanolamine pH 7.8 / 1 mM EDTA / 5 mM DTT / 1 µM leupeptin / 0.5 µg/ml trypsin inhibitor / 0.2 mM PMSF (30 min hypotonic

lysis at 4°C). After addition of NaCl (75 mM final concentration), the homogenate was centrifuged at 75,000 x g for 1 hour at 4°C. The supernatant was mixed with the GSH sepharose 4B for 1 hour at room temperature. The glutathione sepharose 4B was then pelleted by centrifugation at 500 x g for 5 minutes and the supernatant removed. A 10-fold volume of 50 mM Tris-HCl (pH 8.0) / 150 mM NaCl / 2.5 mM $CaCl_2$ / 0.1% 2-mercaptoethanol was added to the glutathione sepharose 4B and mixed for 1 minute. The mixture was centrifuged again at 500 x g for 5 minutes. The supernatant was removed and the glutathione sepharose 4B was washed again in the same way. To elute from the sepharose, the hsGCα1 protein (with the bound hsGCβ1) was cleaved by thrombin from the GST-tag, which remained bound to the glutathione sepharose 4B at the specific cleavage site. Digestion with thrombin was performed in 50 mM Tris-HCl (pH 8.0) / 150 mM NaCl / 2.5 mM $CaCl_2$ / 0.1% 2-mercaptoethanol with 0.25 to 1 units thrombin/ml buffer for 1 or 3 hours at room temperature. The glutathione sepharose 4B (with the GST-tag) was pelleted again by centrifugation at 500 x g for 5 minutes and the supernatant containing the hsGCα1/β1 was removed. Another elution method was performed by addition of 50 mM Tris-HCl (pH 8.0) / 5 mM reduced glutathione and mixing for 30 minutes at room temperature. In this manner, hsGCα1/β1 with the GST-tag bound was removed from the glutathione sepharose 4B. After centrifugation at 500 x g for 5 minutes, the supernatant containing dissolved GST-hsGCα1/β1 was removed.

By the elution with thrombin, a two-fold selectivity is achieved in a single affinity chromatographic step:

1.) Only proteins that have an affinity to reduced glutathione are able to bind.
2.) Of these proteins, only the proteins that are cleaved by thrombin will elute (as cleavage products).

The thrombin can be separated from the sample with a p-aminobenzamidine column to which thrombin binds specifically.

Figure 12 shows the specific enrichment of sGC activity after elution from the GSH sepharose 4B with glutathione compared with the activity in the lysate of infected Sf9 cells.

Figure 14 shows the binding of GST-hsGCα1 to glutathione sepharose 4B and the cleavage of hsGCα1 from the GST-tag by thrombin in an immunoblot.

Figure 15 shows the purification of coexpressed hsGCα1 with GST-tag and hsGCβ1 without GST-tag by affinity chromatography on glutathione sepharose 4B in a Coomassie Brilliant Blue R250-stained SDS-polyacrylamide gel. Upon elution with reduced glutathione, only two bands were visible, which correspond to GST-hsGCα1 (larger product) and hsGCβ1 (smaller product) (detected in an immunoblot). After elution with thrombin (0.25, 0.5, or 1 unit/ml for three hours at room temperature), however, the lower band was identical (hsGCβ1; estimated molecular weight according to the migration in the gel of approximately 70 kDa), whereas the upper band was significantly smaller compared to elution with glutathione (hsGCα1; estimated molecular weight of approximately 80 kDa) because the GST-tag was cleaved off by use of the thrombin elution. This approximately corresponds to the molecular weights of 77.5 kDa for hsGCα1 and 70.5 kDa for hsGCβ1 deduced from the amino acid sequences. In contrast to the elution with reduced glutathione, an additional very small band of approximately 25 kDa was visible after the thrombin elution, which is likely thrombin itself. Thrombin can be removed from the eluate by means of an aminobenzamidine sepharose column to which thrombin binds specifically. Additional bands were not detectable in this experiment.

Example 8

Production of polyclonal rabbit antisera to hsGCα1 and hsGCβ1

Antisera were obtained by immunization of rabbits with synthetic peptides corresponding to sequences from hsGCα1 (Phe-Thr-Pro-Arg-Ser-Arg-Glu-Glu-Leu-Pro-Pro-Asn-Phe-Pro [Figure 22/SEQ ID NO: 5]; amino acids 634-647) and from hsGCβ1 (Lys-Gly-Lys-Lys-Glu-Pro-Met-Gln-Val-Trp-Phe-Leu-Ser-Arg-Lys-Asn-Thr-Gly-Thr-Glu-Glu-Thr [Figure 23/SEQ ID NO: 6] amino acids 593-614) that were coupled to KLH (keyhole limpet hemocyanin) via an additional C-terminal (α1) or N-terminal (β1) cysteine residue. The antisera were affinity-purified with the corresponding peptides coupled to epoxy-activated sepharose (Pharmacia, Freiburg, Germany) according to the manufacturer's instructions.

Example 9

Detection of hsGCα1 and hsGCβ1 in different human tissues by immunoblotting

Human lung tissue was obtained from a tumor-free area of a lung resection, and human cortex and cerebellum were from a normal autopsy. All tissues were immediately frozen in liquid nitrogen and stored at –70°C. The frozen tissues were homogenized in a mortar and double-concentrated, hot SDS stop buffer (130 mM Tris-HCl, pH 6.8 / 16% [v/v] glycerol / 4% [w/v] SDS / 0.025% [w/v] bromphenol blue / 6.5% [v/v] 2-mercaptoethanol) was added to the powder. This was incubated at 95°C for 10 minutes and then centrifuged at 20,000 x g for 20 minutes. The supernatant was used for immunoblotting (for antibodies used, see above).

Expression of both subunits (α1 and β1) was detected in all three tissues (Figure 13). In contrast, expression could not be detected in kidney, liver, and pancreas (data not shown).

Example 10

Construction of recombinant adenoviral hsGC vectors

The cDNAs for hsGCα1 and hsGCβ1 were isolated from the original plasmid with the restriction enzyme EcoRI as 3.0 kb (hsGCα1) and 2.4 kb (hsGCβ3) fragments. The fragments were each inserted into the EcoRI restriction sites of the adenoviral transfer plasmid pZS2 (Figure 16), which contains an adenovirus type 5 sequence (Ad5) with a deletion in the E1 region (ΔE1), followed by an expression cassette with a CMV (cytomegalovirus) promotor/enhancer and a unique XbaI restriction site. hsGCα1-pZS2 and hsGCβ1-pZS2 digested with XbaI were inserted into the XbaI site of the long arm (RR5) of Ad5 (Figure 16). The resulting recombinant adenoviral vectors Ad5CMVhsGCα1 and Ad5CMVhsGCβ1 are replication deficient because they lack the E1 region. To propagate the viruses, 293 cells that express E1 were infected with these viruses. Viral plaques appeared 12-24 hours after the transfection. Viruses from single plaques were purified according to a standard procedure. Plaques containing recombinant viruses (Ad5CMVhsGCα1 or Ad5CMVhsGCβ1) were identified by means of PCR analysis: The plaque material

was freeze-thawed three times, incubated at 37°C for 30 minutes in lysis buffer (16.6 mM ammonium sulfate / 67 mM Tris-HCl pH 6.8 / 6.7 mM $MgCl_2$ / 5 mM 2-mercaptoethanol / 6.7 mM EDTA / 1.7 mM SDS / 50 µg/ml proteinase K), and thereafter heat inactivated for 10 minutes at 85°C. Finally, the DNA was isolated from the lysate with a standard phenol/chloroform extraction and used for PCR analysis.

Example 11

Detection of cGMP formation in EA.hy926 cells after coinfection with the hsGC adenovectors Ad5CMVhsGCα1 and Ad5CMVhsGCβ1

Ten 10-cm cell culture dishes with 'EA.hy926' cells were coinfected with each of the adenovectors Ad5CMVhsGCα1 and Ad5CMVhsGCβ1 at 2×10^{10} pfu (plaque forming units) per dish. After 72 hours, the cells were harvested by adding hypotonic lysis buffer (25 mM triethanolamine pH 7.8 / 1 mM EDTA / 5 mM DTT / 1 µM leupeptin / 0.5 mg/l trypsin inhibitor / 0.2 mM PMSF) and detaching with a cell scraper. The homogenate was centrifuged at 500 x g for 15 minutes and the supernatant was mixed with an equal volume of glycerol and stored at –20°C. The stimulation of hsGC by 100 µM SNP (sodium nitroprusside) was determined by measurement of the basal cGMP level and the cGMP level after treatment with SNP according to the procedure described above (see Example 5). In three samples (A, B, C), a 7-fold to 10.75-fold elevation in the cGMP concentration compared to the basal activity was detectable after SNP stimulation, while no significant elevation was measurable in the control without adenovirus infection (Figure 17).

Example 12

Detection of the cGMP formation in ECV 304 and A10 cells after coinfection with the hsGC adenoviruses Ad5CMVhsGCα1 and Ad5CMVhsGCβ1

Three 10-cm cell culture dishes with 'ECV 304' cells were coinfected with 10^9 - 5×10^{10} pfu (plaque forming units) of each of Ad5CMVhsGCα1 and Ad5CMVhsGCβ1. After 72 hours, the cells were harvested by two washes with PBS buffer (phosphate-buffered saline), addition of hypotonic lysis buffer, and then further treated according to the procedure described in Example 11. The samples were stored and the activity was determined (cGMP content in pmol/mg/min) as described in Example 5. A

significant basal activity (without SNP) and a significant maximal activity (with addition of 100 µM SNP to the assay) was only detectable after the gene transfer of Ad5CMVhsGCα1 and Ad5CMVhsGCβ1 (5 x 10^{10} pfu each) (Figure 26). In control experiments without Ad5CMVhsGCα1 or Ad5CMVhsGCβ1, no significant increase in cGMP formation by SNP was detectable. Qualitatively similar results were obtained in 'A10' vascular smooth muscle cells if these cells were also coinfected with 10^9 – 5 x 10^{10} pfu Ad5CMVhsGCα1 and Ad5CMVhsGCβ1. Here, too, a significant basal activity (without SNP) and a significant maximal activity (with addition of 100 µM SNP to the assay) was only detectable after the gene transfer of Ad5CMVhsGCα1 and Ad5CMVhsGCβ1 (5 x 10^{10} pfu of each) (Figure 26). In control experiments without Ad5CMVhsGCα1 or Ad5CMVhsGCβ1, again no significant increase in cGMP formation by SNP was detectable (Figure 28).

Example 13

Kinetics of cGMP formation in ECV 304 and A10 cells after coinfection with the hsGC adenovectors Ad5CMVhsGCα1 and Ad5CMVhsGCβ1

Three 10-cm cell culture dishes containing either 'ECV 304' or 'A10' cells were coinfected with 5 x 10^{10} pfu (plaque forming units) Ad5CMVhsGCα1 and Ad5CMVhsGCβ1. The cells were harvested, and further treatment, storage, and determination of activity was performed as described in Examples 12, 11, and 5. Whereas at day 0 (before the coinfection), no significant increase in cGMP formation by SNP was detectable, 15 days after the gene transfer of Ad5CMVhsGCα1 and Ad5CMVhsGCβ1, a significant increase in cGMP formation after SNP addition was detectable (Figure 28). Qualitatively similar results were obtained in 'A10' vascular smooth muscle cells if these cells were also coinfected with 5 x 10^{10} pfu Ad5CMVhsGCα1 and Ad5CMVhsGCβ1. Here, 15 days after coinfection, an increase in cGMP formation by SNP was also still detectable (Figure 29). Again, at day 0 (before the coinfection), there was still no significant increase in cGMP formation by SNP. In the same experiments, sGC subunits were detectable after adenoviral gene transfer, but not in noninfected control cells (Figure 30).

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